Chapter-II (Part-A)

Effect of resveratrol on hyperalgesia: antioxidant enzymes, ROS, TNFR1 and ERK activation
7.1 Introduction

ROS is relatively new target of anti-hyperalgesic interventions. Use of natural antioxidants as anti-hyperalgesic agents may have the advantage of intervening hyperalgesia with fewer side effects. Resveratrol, a grape polyphenol has attracted attention for its potent and long lasting anti-nociceptive effects (Pham-Marcou et al., 2008; Torres-Lopez et al., 2002). A number of studies have suggested the anti-nociceptive effect of resveratrol in different animal models of hyperalgesia. Anti-hyperalgesic effect of resveratrol is reported to involve inflammatory as well as opioidergic pathways (Pham-Marcou et al., 2008; Torres-Lopez et al., 2002; Singh and Vinayak, 2016; Gupta et al., 2004). Interestingly, these pathways are linked with concomitant ROS generation (Martínez-Revelles et al., 2013; Wartenberg et al., 2003; Doyle et al., 2010). Therefore, it was hypothesized that anti-hyperalgesic effect of resveratrol might be attributed to its antioxidant property.

Increasing body of evidence suggests analgesic effect of exogenous antioxidants in neuropathic or chronic (Khalil et al., 2004; Park et al., 2006; Kim et al., 2004; Maixner et al., 2016) as well as in inflammatory hyperalgesia (Lee et al., 2007; Wang et al., 2004). Furthermore as described in previous chapter, alleviation of thermal hyperalgesia was found to be associated with improvement of antioxidant defense system and reduction in oxidative stress (Singh and Vinayak, 2015). However, their specific role in initiation, potentiation and maintenance of hyperalgesia is still not understood. Antioxidant enzymes have a crucial role in endogenous antioxidant defense system, apart from non-enzymatic components. ROS may regulate these enzymes at transcriptional or post translational levels (Harris, 1992). Further, various antioxidant enzymes may be modulated in different ways (Shull et al., 1991). We have reported a tissue specific alteration in antioxidant enzymes in paw skin and spinal cord during initiation of inflammatory pain (Singh and Vinayak, 2015).

Enzymatic antioxidant defense system includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Both isozymes of SOD, Cu-Zn SOD and Mn-SOD protect cells from oxidative damage at initial step by scavenging superoxide radicals. Hydrogen peroxide, a byproduct of SOD may further be converted into water by either catalase or GPx (Khan et al., 2013).
ROS regulates a number of downstream signaling pathways leading to inflammation and hyperalgesia. Pro-inflammatory cytokine TNF-α is a major cytokine in inflammatory soup which has a lead role in activating a cascade of other cytokines in pain signaling (Rittner et al., 2005). TNF-α causes increase in the expression of TRPV1, which is a cation channel having a major role in nociceptive signaling. TNFR1 and TRPV1 are reciprocally regulated in nociceptive neurons, mediated via ERK activation and ROS generation (Hensellek et al., 2007; Ma et al., 2009). Further, ROS may activate ERK and other MPAKs (Hensley et al., 2000). All the three MAPK pathways are reported to be involved in hyperalgesia (Ji et al., 2009). Therefore, it may be hypothesized that ROS sensitizes nociceptors by MAPK activation, and antioxidant resveratrol should be able to reduce the signaling pathway towards anti-hyperalgesic action.

Therefore in the present study, the hypothesis was tested by investigating the role of antioxidant enzymes, ROS and downstream targets TNFR1-ERK in initiation and maintenance of hyperalgesia and the impact of resveratrol treatment. Early phase (6h after CFA administration) and late phase (48h after CFA administration) were selected to represent initiation and maintenance of hyperalgesia. Paw skin and spinal cord were used to understand the molecular alteration at peripheral and central levels, respectively.

7.2 Results

All biochemical and molecular analysis was performed in paw skin and lumbar region (L4-L6) of spinal cord representing peripheral and central sites of nociception. Initial hyperalgesic behavior is generated by peripheral sensitization of neurons.

7.2.1 Effect of resveratrol on thermal hyperalgesia

Paw withdrawal latency of rats is inversely proportional to hyperalgesia. The hyperalgesic effect was evidenced at different time intervals from 2h to 48h after CFA administration. As compared with PWL of control rats (N), CFA induced rats (C) showed significant decrease by approximately 35% (p<0.01), 48% (p<0.001), 50% (p<0.001), 54% (p<0.001) and 50% (p<0.001) at 2h, 6h, 12h, 24h and 48h, respectively. DMSO did not show any significant difference.

Resveratrol showed anti-hyperalgesic effect from 6h to 48h (Fig 7.1). The PWL was gradually elevated in resveratrol treated rats (CR) with increasing time period, which was approximately 20% (p<0.05), 38% (p<0.05), 47% (p<0.01) and 41% (p<0.01) high at 6h,
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12h, 24h and 48h respectively, as compared to DMSO injected rats (CD). The pattern of behavioral hyperalgesia was similar as found with curcumin treatment. The result indicates that anti-hyperalgesic action of resveratrol was initiated at 6h. Resveratrol treatment caused almost complete reversal of hyperalgesia by 48h. Therefore, two time points 6h and 48h after CFA administration were selected for further study, representing early phase and late phase of hyperalgesia respectively.

Figure 7.1 Effect of resveratrol on PWL in hyperalgesic rats
Anti-hyperalgesic activity in terms of PWL by hot plate test in normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats at different time intervals after CFA injection. Each group included 6-9 rats. Results represent mean±S.E.M. obtained from three different sets of experiments. # Denotes significant difference as compared with N group (*p<0.05, **p<0.01, ###p<0.001) *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001).

7.2.2 Effect of resveratrol on ROS level during early and late phase
The elevation in ROS level during early and late phase of hyperalgesia showed tissue specificity. ROS level in paw skin was progressively elevated from early phase (approximately by 129%) to late phase (approximately by 200%) as compared to normal (Fig. 7.2a). Whereas in spinal cord, the level was increased by approximately 33% in early phase and remained constant in late phase (Fig. 7.2b). Resveratrol treatment could bring down the ROS level up to almost normal level during early phase (6h) in both tissues. Similarly, it was significantly decreased by resveratrol treatment during late phase (48h). However, it remained approximately 37% and 17% higher than the normal level in paw skin and spinal cord, respectively (Fig. 7.2a, 7.2b).
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Figure 7.2 Effect of resveratrol on ROS level in paw skin and spinal cord during early and late phases
Level of ROS in (a) paw skin and (b) spinal cord of normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats during early (6h) and late phase (48h). ROS level was measured by oxidative conversion of \( \text{H}_2\text{DCFDA} \) to fluorescent DCF. Data are presented in terms of fluorescence intensity/mg protein. Results represent mean\( \pm \)S.E.M. obtained from three different sets of experiments. * denotes significant difference (*\( p<0.05, **p<0.01, ***p<0.001 \)), ‘ns’ denotes not significant difference.

7.2.3 Effect of resveratrol treatment on antioxidant enzymes
Effect of resveratrol on antioxidant defense system was evaluated by monitoring antioxidant enzyme activities in hyperalgesic rats. The primary antioxidant enzymes (CAT, SOD and GPX) were assayed by activity gel in paw skin and spinal cord of rats. The activities of all the enzymes showed tissue specificity in early and late phases.

7.2.3.1 Catalase
In case of paw skin, the activity of catalase was found to be reduced after 6h of CFA injection approximately by 47\% (\( p<0.001 \)) as compared to the normal rats; however resveratrol treatment caused enhancement in the activity upto approximately normal level (Fig 7.3a). In spinal cord, the variation pattern was just opposite. The activity was increased by 27\% (\( p<0.001 \)) as compared to normal rats, which was brought back to approximately normal level after resveratrol treatment (Fig 7.3b). At 48h, catalase activity in paw skin was found to be unaltered in CFA induced rats (C) as well as after resveratrol treatment (CR) (Fig 7.3c). The variation pattern was again found to be
different in case of spinal cord. The activity of catalase was enhanced in CFA induced rats by approximately 58% (p<0.001) which was normalized by resveratrol treatment (Fig 7.3d).

![Figure 7.3](image)

**Figure 7.3 Effect of resveratrol treatment on catalase activity in hyperalgesic rats**

Catalase activity in (a, c) paw skin and (b, d) spinal cord of hyperalgesic rats during early phase (6h) & late phase (48h) of hyperalgesia. Activity was compared in normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats. Results represent mean±S.E.M. obtained from three different sets of experiments. Each group included 6 rats. # denotes significant difference as compared with N group (#p<0.05, ##p<0.01, ###p<0.001). *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001).

### 7.2.3.2 Superoxide dismutase (SOD)

The variation pattern of SOD activity in CFA injected (C) as well as resveratrol treated (CR) rats was similar to that of catalase activity in both tissues at 6h. The activity of SOD was reduced approximately by 37% (p<0.001) in paw skin after 6h of CFA injection as compared to normal, and was enhanced upto approximately 85% (p<0.05) after
Resveratrol treatment (Fig 7.4a). In case of spinal cord, SOD activity was increased by 20% (p<0.001) as compared to normal rats. Resveratrol treatment brought the activity up to almost normal level (Fig 7.4b). However, during late phase the activity of SOD did not show any change in CFA induced and resveratrol treated rats in both the tissues (Fig 7.4c, 7.4d).

**Figure 7.4 Effect of resveratrol treatment on SOD activity in hyperalgesic rats**

SOD activity in (a, c) paw skin and (b, d) spinal cord of hyperalgesic rats during early phase (6h) & late phase (48h) of hyperalgesia. Activity was compared in normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats. Results represent mean±S.E.M. obtained from three different sets of experiments. Each group included 6 rats. *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001).

### 7.2.3.3 Glutathione peroxidase (GPx)

Variation pattern of GPx activity was similar to SOD activity in CFA injected (C) as well as resveratrol treated (CR) rats in both tissues at both time points. During early phase, the activity of GPx in paw skin was reduced approximately by 31% (p<0.001) after CFA.
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Injection, and resveratrol treatment caused enhanced activity upto approximately 86% (p<0.01) as compared to normal (Fig 7.5a); whereas in case of spinal cord GPx activity was increased by 30% (p<0.001) in CFA injected (CR) rats and resveratrol treatment brought back the activity almost upto normal level (Fig 7.5b). However no change was observed in the activity of GPx in CFA injected (C) as well as resveratrol treated (CR) rats in late phase (Fig 7.5c, 7.5d).

Figure 7.5 Effect of resveratrol treatment on GPx activity in hyperalgesic rats
GPx activity in (a, c) paw skin and (b, d) spinal cord of hyperalgesic rats during early phase (6h) & late phase (48h) of hyperalgesia. Activity was compared in normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats. Results represent mean±S.E.M. obtained from three different sets of experiments. Each group included 6 rats. # denotes significant difference as compared with N group (# p<0.05, ## p<0.01, ### p<0.001). *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001).
7.2.4 Effect of resveratrol on TNFR1-ERK signaling

Activation of early gene ERK has been recently considered as neuronal marker of pain (Gao and Ji, 2009). Therefore, modulation of ERK activation was checked to analyze the anti-hyperalgesic potential of resveratrol. Further, the effect of resveratrol on TNFR1 level was examined to evaluate TNFR1 mediated ERK signaling, which is known to be involved in hyperalgesia (Hensellek et al., 2007).

7.2.4.1 Level of pERK/ERK

Activation of ERK is marked by its phosphorylation. Therefore, ERK activation was measured indirectly in terms of the ratio of pERK/ERK, which was found to be significantly high in CFA injected rats (C). The pERK/ERK ratio was increased by approximately 51% \((p<0.001)\) and 49% \((p<0.01)\) respectively at 6h and 48h in spinal cord (Fig 7.6a, 7.6b). Resveratrol treatment caused a decrease in ERK phosphorylation up to approximately normal level at both the time points.

7.2.4.2 Level of TNFR1

Initial pro-inflammatory cytokine TNF-\(\alpha\) signals for hyperalgesia via TNFR1. TNFR1 level was found unaltered in CFA injected (C) as well as resveratrol treated (CR) rats at both time points, as compared to normal rats (Fig 7.6c, 7.6d). As resveratrol treatment did not show modulation of TNFR1 level in spite of regulation of ROS level and ERK activation, it may be suggested that ERK activation by ROS is not totally dependent on TNFR1.
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Figure 7.6 Effect of resveratrol on ERK phosphorylation and TNFR1 level in spinal cord
Level of pERK after (a) 6h and (b) 48h, and level of TNFR1 after (c) 6h and (d) 48h in spinal cord of normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats. Results represent mean±S.E.M. obtained from three different sets of experiments. Each group included 6 rats. * denotes significant difference as compared with N group (p<0.05, **p<0.01, ***p<0.001). Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001).

7.3 Discussion

PWL is inversely proportional to the extent of hyperalgesia. PWL test showed that hyperalgesia was fully developed at 6h and was maintained up to 48h after CFA induction. Therefore, hyperalgesia at 6h and 48h are categorized as early and late phase respectively. Anti-hyperalgesic effect of resveratrol was shown in both phases.

Generation of ROS has been earlier correlated with development of hyperalgesia (Viggiano et al., 2005). We have traced out the pattern of ROS generation during early and late phases at peripheral as well as central site in CFA induced hyperalgesia. Differential pattern of changes in ROS level was found at peripheral and central sites.
The level of ROS was progressively elevated during early and late phases in paw skin. However in spinal cord, the increase in early phase did not continue further up to late phase. Development of hyperalgesic response is reported to be associated with modulation in activities of antioxidant enzymes; however pattern of alteration varies with different antioxidant enzymes during neuropathic chronic pain. Naik et al. (2006) have demonstrated a decreased SOD activity and unaltered catalase activity in sciatic nerve in CCI model of neuropathic pain. Guedes et al. (2006) have shown decreased activity of catalase and SOD in spinal cord of SNT model of neuropathic pain. In contrast, Varija et al. (2008) have reported increased SOD and GPx activity, and decreased catalase activity in different tissues of silver wire ligated neuropathic pain model. We have earlier demonstrated a decreased activity of antioxidant enzymes in paw skin and increased activity in spinal cord in early phase of CFA induced hyperalgesic rats (Singh and Vinayak, 2015). Discrepancies in these reports might be a result of differences in animal models, tissues or time of estimation of enzyme activities. On the basis of these possibilities, we planned to analyze the activities of antioxidant enzymes at peripheral (paw skin) as well as central level (spinal cord); and at early as well as late phase of CFA induced hyperalgesia. Differential pattern of changes in antioxidant enzymes in paw skin and spinal cord at early and late phases matches with changes in ROS level.

Synthetic antioxidant mimetic compounds or purified natural antioxidants are known to show anti hyperalgesic effects (Wang et al., 2004; Mittal et al., 2009). However, modulation of endogenous antioxidant enzymes is not well studied in inflammatory pain. In our previous study, modulation of antioxidant defense system was suggested to be an early event of CFA induced hyperalgesia. In the present study, following the earlier pattern the activities of antioxidant enzymes SOD, catalase and GPx were attenuated in paw skin and induced in spinal cord of hyperalgesic rats at 6h. Decreased activity in paw skin and increased activity of enzymes in spinal cord is correlated with high level of ROS in paw skin as compared to spinal cord at early phase. The level of ROS was elevated by approximately 85% in paw skin and 33% in spinal cord during early phase. Opposite effects on activities of antioxidant enzymes in paw skin and spinal cord may be explained on the basis of differential response of these enzymes for difference in the level of ROS as described in previous chapter. The activities of these enzymes were brought back
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towards normal in both tissues after resveratrol treatment. Thus, anti-hyperalgesic effect of resveratrol might be correlated with antioxidant activity.

The activity of only Cu-Zn SOD was detected by in-gel activity assay in both the tissues during both phases. Cu-Zn SOD was confirmed after comparison with previous in-gel activity assays in our lab (Das and Vinayak, 2014), although presence of Mn-SOD cannot be ruled out. Nonetheless, this result suggests a major role of Cu-Zn SOD in CFA induced inflammatory hyperalgesia at peripheral and central sites. Earlier reports are available showing involvement of Mn-SOD in inflammatory hyperalgesia (Wang et al., 2004) and neurodegeneration (Noack et al., 1998). However, sufficient information is not available on role of Cu-Zn SOD.

In-gel activity staining of GPx indicated modulation in activity of GPx-1 isozyme in hyperalgesia. GPx-1 is a selenoprotein having substrate preference for hydrogen peroxide over other peroxides (Das and Vinayak, 2012). Therefore, modulation in GPx-1 along with catalase suggests an important role of hydrogen peroxide during hyperalgesia.

The effect of ROS on activities of antioxidant enzymes was different in the late phase of hyperalgesia. Despite of high level of ROS, the activities of antioxidant enzymes were brought almost equal to that of normal rats in paw skin during late phase. In case of spinal cord, only catalase activity was increased in CFA induced rats, which is decreased almost upto normal level after resveratrol treatment. There is no report available about differential modulation of antioxidant enzymes during early and late phase of hyperalgesia. Only few reports are available about the regulation in antioxidant enzyme activities in spinal cord under different conditions of neuronal damage or oxidative stress. Kaynar et al. (1994) have reported no change in level of SOD, Catalase and GPX enzymes upto 24h of spinal cord injury. However, when analyzed beyond time limitation, catalase activity was found elevated significantly with no change in SOD and GPX activities (Kaynar et al., 1994). Our result follows similar pattern of variation showing elevated activity in catalase during late phase of hyperalgesia with almost no change in other antioxidant enzymes. Alternatively, apart from primary antioxidant enzymes, cysteine-based antioxidant enzymes of peroxiredoxin and thioredoxin systems might be important in maintaining SOD and GPX activity unaltered by regulation of local ROS level in late phase. Peroxiredoxins are known to exert a neuroprotective effect against
ROS in several models of neurodegeneration (Soriano et al., 2008). Modulation in the thioredoxin system in both animal models and in the postmortem brains of human patients is reported to be associated with the most common neurodegenerative disorders, showing its important role towards defense against oxidative stress (Silva-Adaya et al., 2014). Similar mechanism might be involved in inflammatory hyperalgesia. However, more definitive experiments are needed to understand the mechanism of differential response by antioxidant enzymes.

TNF-α has a lead role in activating a cascade of other cytokines in pain signaling (Rittner et al., 2005). TNFR1 is involved in TNF-α mediated activation of TRPV1 channels in nociceptors (Hensellek et al., 2007). ROS mediated TNFR1 upregulation is reported in cultured DRG neurons; however the molecular mechanism is not known (Ma et al., 2009). Moreover, effect of ROS on spinal TNFR1 still remains unrevealed. Present study shows no significant change in TNFR1 level during CFA induced hyperalgesia.

Spinal ERK is a player between reciprocal regulation of TNFR1 and TRPV1, and its activation is now considered as a hallmark of hyperalgesia (Hensellek et al., 2007; Ma et al., 2009; Gao and Ji, 2009). ROS regulates TNF-α mediated signaling and ERK activation (Hensley et al., 2000). Our results suggest that ROS acts on downstream mediator ERK but not on TNFR1. Further, ROS may be activating ERK-MAPK by inhibition of PTPs as reported earlier (Lee and Esselman, 2002). Since resveratrol treatment could decrease the ROS level without affecting antioxidant enzymes, it may be proposed that reversal of ERK phosphorylation by resveratrol might be due to direct quenching of ROS, rather than involvement of antioxidant enzymes and TNFR1. Direct scavenging of hydroxyl and superoxide radicals by resveratrol is reported earlier (Leonard et al., 2003); however more definitive experiments are needed to confirm this hypothesis.

7.4 Summary

Present study indicates important role of antioxidant defense system during early phase of hyperalgesia at peripheral as well as central level; however its role is restricted to catalase activity at central level in late phase of hyperalgesia. Anti-hyperalgesic effect of resveratrol is exhibited by modulation of ROS and ERK activity in both phases.
Chapter-II (Part-B)

Effect of resveratrol on hyperalgesia: pro-inflammatory cytokines and enzymes
8.1 Introduction

A tissue injury leads to secretion of inflammatory mediators by infiltrating as well as local defense cells. Inflammation-associated changes in the microenvironment of nociceptors result in hypersensitivity of neurons (Dray, 1995). TNF-α is a prototypic pro-inflammatory cytokine which initiates a cascade of cytokines and growth factors during an inflammatory response (Dray, 1995; Rittner et al., 2005). Cytokines TNF-α, IL-6 and IL-1β activate COX-2 which results in inflammatory hyperalgesia via prostaglandins (Salvemini et al., 1996; Rittner et al., 2005). Further, inflammatory cells release nitric oxide (NO) by activation of inducible nitric oxide synthase (iNOS), which induces hyperalgesia by inhibition of antioxidant enzymes and activation of PKCs (Salvemini et al., 2011). Exposure of nociceptors to inflammatory mediators leads to peripheral sensitization, which is restricted to the site of tissue injury (Hucho et al., 2007), whereas central sensitization results from changes in the properties of neurons in the CNS. The early phase of hyperalgesia is initiated by peripheral sensitization; however the late phase is mainly due to central sensitization. Therefore, specific targets should be specified for treatment of peripheral and central hyperalgesia (Latremoliere et al., 2009).

In previous chapter, a correlation has been found between antioxidant and anti-hyperalgesic properties of resveratrol. ROS modulates several inflammatory mediators like pro-inflammatory cytokines and pro-inflammatory enzymes. Therefore, present study is focused to examine the modulation of inflammatory cytokines IL-1β, TNF-α, IL-6 and enzymes COX-2 and iNOS by resveratrol in paw skin and spinal cord during early as well as late phase of hyperalgesia.

8.2 Results

8.2.1 Effect of resveratrol on c-Fos expression in spinal dorsal horn

Early gene c-Fos is a well known neuronal marker of hyperalgesia (Harris, 1998). Its expression was compared between ipsilateral and contralateral side of spinal dorsal horn of CFA induced rats. When checked by immunohistochemistry (IHC), CFA administration unilaterally up-regulated the expression of c-Fos only in the ipsilateral side (Fig 8.1). Further, the number of c-Fos positive cells was compared in all the four groups. The number of c-Fos positive cells was high in CFA induced rats (C) as compared to normal (N). There was no apparent difference in number of c-Fos positive
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cells in DMSO treated rats (CD) as compared to group C. However, resveratrol treatment caused a decrease in the number of c-Fos positive cells as compared to CD group (Fig 8.2). Thus, the anti-hyperalgesic effect of resveratrol shown by PWL test was confirmed by neuronal marker c-Fos. Anti-hyperalgesic effect of resveratrol in terms of c-Fos expression is in agreement with an earlier published report (Bazzo et al., 2013).

![Image](image_url)

Figure 8.1 CFA evoked unilateral up-regulation of c-Fos immuno-reactivity in spinal dorsal horn
c-Fos expression is shown by dark stained cells in dorsal horn of L4-L6 section of spinal cord. (ii) whole section in low magnification, (i) contralateral & (iii) ipsilateral dorsal horn in high magnification. Scale bar represents 125μm.
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Figure 8.2 Immuno-histochemical analysis of c-Fos in spinal dorsal horn
C-Fos expression in ipsilateral dorsal horn in normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats. Number of c-Fos positive cells was considered irrespective of intensity of stain. Arrow shows c-Fos positive cells. Scale bar represents 125μm.

8.2.2 Effect of resveratrol on inflammatory mediators during early and late phase of hyperalgesia
Role of inflammatory mediators is well established in hyperalgesia. However, their specific role in early and late phase of hyperalgesia is still not known. Therefore, levels of pro-inflammatory enzymes and cytokines were compared in the two phases.

8.2.2.1 Pro-inflammatory enzymes COX-2 and iNOS
COX-2 and iNOS are inflammatory enzymes involved in pain sensation (Rittner et al., 2005). Protein expression of COX-2 was found to be significantly high in paw skin as well as in spinal cord at 6h and 48h after CFA administration. The level of COX-2 in paw skin was approximately 14 times (p<0.001) high in CFA administered rats at 6h (Fig 8.3) as compared to normal rats, whereas at 48h it was only 1.5 times high (p<0.01) (Fig 8.4), suggesting a major contribution of peripheral COX-2 in the initial phase. However in case of spinal cord, COX-2 level was approximately upto 1.2 times (p<0.01) higher at 6h
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(Fig 8.3) which was further increased up to 4.7 times (p<0.001) at 48h (Fig 8.4), which indicates major role of COX-2 at central site during late phase. The differential role of COX-2 at peripheral and central level may be correlated with its involvement during initiation and maintenance of hyperalgesia.

Resveratrol treatment caused down-regulation in the level of COX-2 at early as well as late phase in both tissues. The level of COX-2 was decreased in paw skin up to approximately 48% (p<0.001) and 51% (p<0.01) by resveratrol treatment at 6h and 48h, respectively, as compared to CD group. Similarly in spinal cord, the level of COX-2 was decreased up to approximately 49% (p<0.001) and 46% (p<0.01) at 6h (Fig 8.3) and 48h (Fig 8.4) respectively, as compared to CD group.

Further, iNOS level was checked to analyze its contribution in hyperalgesia and the effect of resveratrol. Interestingly, iNOS activity was restricted to paw skin only as no detectable level of iNOS was found in spinal cord at any time point. CFA administration induced the iNOS level at both time points in paw skin (Fig 8.3, Fig 8.4). Restricted expression of iNOS in paw skin suggests its role in development of hyperalgesia at peripheral level. Resveratrol treatment brought down the level of iNOS towards normal at both time points in paw skin indicating its anti-hyperalgesic activity.
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Figure 8.3 Effect of resveratrol on level of COX-2 and iNOS in paw skin and spinal cord of hyperalgesic rats in early phase

(i) Paw skin

(ii) Spinal cord

(iii)

(iv)

(v)

(iii)

(iv)

(v)

Denotes significant difference as compared with N group (*p<0.05, **p<0.01, ***p<0.001)

Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001)
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8.2.2.2 Pro-inflammatory cytokines

TNF-α, IL-1β and IL-6 were enhanced in paw skin of CFA induced rats in both phases, whereas their differential regulation was observed in spinal cord in a time specific manner.

8.2.2.2.1 Effect in paw skin

Pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were found to be elevated in CFA induced rats at both time points as compared to normal rats. The level was approximately upto 1.7 times (p<0.05), 3 times (p<0.001) and 7 times (p<0.001) higher respectively at 6h (Fig 8.5); and approximately upto 1.2 times (p<0.05), 4.6 times (p<0.001) and 1.2
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times (p<0.05) respectively at 48h (Fig 8.6). Resveratrol treatment did not show any significant difference in the level of these cytokines at both time points (Fig 8.5, Fig 8.6).

Figure 8.5 Effect of resveratrol on level of pro-inflammatory cytokines in paw skin of hyperalgesic rats in early phase
(i) TNF-α, (ii) IL-1β and (iii) IL-6 in paw skin of normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats after 6h of CFA injection. Each group included 4 rats. # Denotes significant difference as compared with N group (*p<0.05, **p<0.01, ###p<0.001) *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001)

Figure 8.6 Effect of resveratrol on level of pro-inflammatory cytokines in paw skin of hyperalgesic rats in late phase
(i) TNF-α, (ii) IL-1β and (iii) IL-6 in paw skin of normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats after 48h of CFA injection. Results represent mean±S.E.M. obtained from three different sets of experiments. Each group included 4 rats. # Denotes significant difference as compared with N group (*p<0.05, **p<0.01, ###p<0.001) *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001)
8.2.2.2.2 Effect in spinal cord
The variation pattern of pro-inflammatory cytokines in spinal cord did not follow the pattern found in paw skin. There was no significant difference in level of TNF-α, IL-1β and IL-6 in normal, CFA induced as well as resveratrol treated rats during early phase (Fig 8.7). The results suggest that there is no glial activation in spinal cord during early phase of hyperalgesia as suggested in previous chapter. However during late phase, the level of cytokines TNF-α, IL-1β and IL-6 was increased in CFA administered rats approximately up to 1.5 times ($p<0.05$), 2.2 times ($p<0.05$) and 2.3 times ($p<0.01$) respectively as compared to normal rats. Resveratrol treatment decreased the levels of all three cytokines almost up to normal (Fig 8.8).

![Figure 8.7](image)

Figure 8.7 Effect of resveratrol on level of pro-inflammatory cytokines in spinal cord of hyperalgesic rats in early phase
(i) TNF-α, (ii) IL-1β and (iii) IL-6 in spinal cord of normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats after 6h of CFA injection. Results represent mean±S.E.M. obtained from three different sets of experiments. No significant difference was found between all four groups.
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Figure 8.8 Effect of resveratrol on level of pro-inflammatory cytokines in spinal cord of hyperalgesic rats in late phase
(i) TNF-α, (ii) IL-1β and (iii) IL-6 in skin of normal (N), CFA induced (C), CFA+DMSO treated (CD) and CFA+resveratrol treated (CR) rats after 48h of CFA injection. Results represent mean±S.E.M. obtained from three different sets of experiments. Each group included 4 rats. # Denotes significant difference as compared with N group (#p<0.05, ##p<0.01, ###p<0.001) *Denotes significant difference as compared with CD group (*p<0.05, **p<0.01, ***p<0.001)

8.3 Discussion

COX-2 is an inducible enzyme which plays a key role in biosynthesis of prostaglandins from arachidonic acid. Prostaglandins are important for a large number of pathological conditions where they promote inflammation, swelling, pain etc. Role of nitric oxide producing enzymes cNOS and iNOS has also been suggested in recent literature. Our results indicate anti-hyperalgesic activity of resveratrol via down-regulation of COX-2 in both tissues and at both phases. Protein expression of iNOS was restricted to peripheral site, which was attenuated by resveratrol at early (6h) as well as late (48h) phase. COX-2 and iNOS may be differentially regulated in the two tissues (Zhua et al., 2012). Modulation of pro-inflammatory enzymes by resveratrol supports its anti-nociceptive effect at early as well as late phase. A recent report suggests down-regulation of mRNA expression of COX-2 and iNOS by resveratrol (Dung et al, 2015). Relative specificity of resveratrol for COX-2/COX-1 has been debated for a long time (Subbaramaiah et al., 1998; Szewczuk et al., 2004). The anti-nociceptive effect of resveratrol is proposed to be related to inhibition of COX-1 activity by Torres-Lopez et al. (2002). On the other hand,
Pham-Marcou et al. (2008) have proposed involvement of COX-2 in resveratrol mediated anti-hyperalgesic action.

Inflammation is associated with a rapid increase in the level of pro-inflammatory cytokines (Basbaum et al., 2009). Several inflammatory cells as macrophages, neutrophils, monocytes and resident keratinocytes secrete cytokines at the peripheral level (Basbaum et al., 2009). These cytokines induce pain by activating peripheral terminals of nociceptors directly or indirectly (Rittner et al., 2005). It is interesting to note that resveratrol was effective in decreasing the inflammatory enzymes COX-2 and iNOS but not the cytokines TNF-α, IL-1β and IL-6 in paw skin during early as well as late phase. There are few reports suggesting dissociation of COX-2 regulation from pro-inflammatory cytokines. Ejima et al. (2003) have shown similar levels of pro-inflammatory cytokines in wild type and COX-2 knockout mice. Similarly, the level of plasma TNF-α was not affected by selective pharmacological inhibition of COX-2 in a lipopolysaccharide (LPS) induced murine endotoxemia model (Reddy et al., 2001). The present results suggest that resveratrol may be directly affecting the downstream mediators of cytokines i.e. COX-2 and iNOS rather than cytokines in paw skin. The regulation of pro-inflammatory cytokines and enzymes by resveratrol is not well documented in hyperalgesia. However, there are indirect reports showing that resveratrol did not affect secretion of pro-inflammatory cytokines in peritoneal macrophages in different pathological conditions (Park et al., 2014; Wadsworth et al., 1999).

Central sensitization in inflammatory hyperalgesia is often associated with glial activation and secretion of cytokines in spinal cord (Sheu et al., 2013). In the present study, differential secretion of cytokines was observed in spinal cord at early (6h) and late (48h) phase. Cytokines TNF-α, IL-1β and IL-6 remain at basal level during early phase of CFA induced hyperalgesia as well as after resveratrol treatment. However, time specific secretion of these cytokines and its down regulation by resveratrol was noticed during late phase. We have earlier found no change in the level of spinal TNFR1 in both phases. The result indicates that resveratrol inhibits the TNF-α signaling in spinal cord during late phase without any alteration in total TNFR1 level. Significant increase in the levels of cytokines suggests activation of glial cell during late phase only. These results
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further support an inhibitory effect of resveratrol on glial activation in spinal cord during late phase. These findings are in agreement with earlier indirect reports showing neuroprotective effect of resveratrol by inhibition of glial cell activation (Sheu et al., 2013; Zhang et al., 2010).

8.4 Summary

The results demonstrate differential regulation of pro-inflammatory cytokines and enzymes in time specific and tissue specific manner as specified by (1) limited protein expression of iNOS in hyperalgesia and its regulation by resveratrol in peripheral tissue (2) time specific (late phase) secretion of pro-inflammatory cytokines and its modulation by resveratrol at central level (3) down-regulation of COX-2 by resveratrol in both early and late phases at the peripheral and central levels. The study suggests anti-hyperalgesic effect of resveratrol at both peripheral and central sites in early and late phases. Overall effect of resveratrol in hyperalgesic rats is depicted in the following figure-

(A)

Peripheral

- ROS → Peripheral sensitization
- CAT ↓, SOD ↓, GPx ↓
- TNF-α ↑
- IL-1β ↑
- IL-6 ↑
- COX-2 ↑
- iNOS ↑

Central

- ROS → Central sensitization
- CAT ↑, SOD ↑, GPx ↑
- TNF-α ↔
- IL-1β ↔
- IL-6 ↔
- pERK ↑
- TNFR1 ↔
- COX-2 ↑
- iNOS X

Resveratrol

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(B)

Peripheral

ROS ↓
Peripheral sensitization

CAT ↔
SOD ↔
GPx ↔

TNF-α ↑
IL-1β ↑
IL-6 ↑
COX-2 ↑
iNOS ↑

Resveratrol

Hyperalgesia

Central

ROS ↓
Central sensitization

CAT ↑
SOD ↔
GPx ↔

TNF-α ↑
IL-1β ↑
IL-6 ↑
pERK ↑
TNFR1 ↔
COX-2 ↑
iNOS X

Late phase

Schematic representation of anti-hyperalgesic action of resveratrol in (A) early phase and in (B) late phase